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RESEARCH PAPER

Preparation of Rotigotine-Loaded Microspheres and Their Combination Use with L-DOPA to Modify Dyskinesias in 6-OHDA-Lesioned Rats

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ABSTRACT

Purpose To prepare rotigotine loaded microspheres (RoMS) to achieve continuous dopaminergic stimulation (CDS) for the treatment of Parkinson's disease (PD) and investigate both the therapeutic benefit and inducibility of AIMs of administration of RoMS combination with L-DOPA in 6-OHDA-leisioned rats.

Methods Rotigotine was encapsulated into poly(lactic-co-glycolic acid) (PLGA) microspheres by an oil-in-water emulsion solvent evaporation technique. *In vitro* characteristics and *in vivo* pharmacokinetics of RoMS either in rat blood or brain (by microdialysis) were investigated. Contraversive rotations and AIMs were observed to investigate the therapeutic benefit and the propensity to induce dyskinesia of RoMS or RoMS combination with L-DOPA in 6-OHDA-lesioned rats.

Results RoMS displayed continuous-release characteristics of rotigotine in animals and exhibited a steady efficacy lasted for 2 weeks in 6-OHDA-lesioned rats. No significant difference of the therapeutic benefit between the treatment of RoMS and pulsatile L-DOPA combination and mono L-DOPA was found. While the dyskinesia was significantly decreased with the treatment of RoMS and pulsatile L-DOPA combination compared to mono L-DOPA.

Conclusions RoMS could supply an alternative of CDS for the treatment of PD and the study indicates a potential advantage of RoMS for the treatment of mild and advanced PD patient in combination with L-DOPA.

KEY WORDS combination with L-DOPA · continuous dopaminergic stimulation · dyskinesia · Parkinson's disease · rotigotine loaded microspheres

ABBREVIATIONS

AIMs	abnormal involuntary movements
Apo	apomorphine
CDS	continuous dopaminergic stimulation
EE	encapsulation efficiency
im	intramuscular injection
ip	intraperitoneal injection
L-DOPA	levodopa
LID	levodopa induced dyskinesias
PD	Parkinson's disease
PLGA	poly(lactic-co-glycolic acid)
po	per os
RoMS	rotigotine loaded microspheres

INTRODUCTION

Parkinson's disease (PD) is a slowly progressive, degenerative disorder of the central nervous system, which leads to expression of motor symptoms characterized by resting

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tremor, muscular rigidity and bradykinesia (1,2). Therapy with levodopa (L-DOPA) has been proved to be the most effective symptomatic treatment for PD for over 30 years (3). However, long-term treatment with L-DOPA results in the development of disabling and troublesome motor complications called dyskinesias, a movement disorder characterized by abnormal involuntary movements (AIMs) and usually designated as L-DOPA induced dyskinesias (LID) (4,5). It is hard to control and limit the utility of L-DOPA (6), hence, an effective therapy of dyskinesia is needed for all clinicians caring for advanced PD patients.

Substantial studies have implicated a relationship between intermittent or pulsatile stimulation of striatal dopamine receptors and LID in PD (7–9). These motor complications can be improved by long-acting dopaminergic drugs which can provide continuous receptor stimulation (6,10). L-DOPA and dopamine agonists have inspired the therapeutic concept of continuous dopaminergic stimulation (CDS), which has been developed and received considerable attention for treatment of PD (11,12). Theoretically, CDS can provide a constant supply of dopamine or a dopamine agonist to striatal dopamine receptors which should mimic the continuously stimulation of dopaminergic receptors in the normal manner to benefit the PD patients (12–14). The therapeutic concept of CDS has played a prominent role in the treatment of early PD which can delay the onset of dyskinesia (3,15). Dopamine agonists with long half-life, for instance, ropinirole or bromocriptine, show less potential to induce dyskinesia compared to L-DOPA (16,17). In addition, less dyskinesia is going to be developed by continuous infusion of a short half-life dopamine agonist than that by repeated injection of the same agent (18). Moreover, continuous infusion therapies with apomorphine and L-DOPA have been demonstrated to decrease the intensity of established dyskinesia which suggests that CDS may also benefit the treatment of mild and advanced PD (19,20). However these therapies with infusion devices require higher levels of patient commitment as well as maintenance of drug delivery devices.

Currently available CDS treatment options include duodenal infusion of L-DOPA (Duodopa®) and rotigotine transdermal delivery system (Neupro®). The beneficial effect of Duodopa® is that it can provide continuous and steady L-DOPA plasma levels resulting in significant reduction of dyskinesia. It is considered to be an effective treatment for advanced PD patients with motor complications. However, duodenal L-DOPA administration has several disadvantages. First, a small tube is required to be implanted to the duodenum surgically. Secondly, the accompanying pump devices may be cumbrous. Finally, high cost may be a limiting factor (21). Neupro® (once-daily rotigotine transdermal patch) provided 24-h continuous drug levels that would improve efficacy and reduce motor complications compared with other currently available products. However Neupro® skin patches were withdrawn from the US market in April 2008 because of drug crystallization, the rotigotine crystals formed on the patches were not absorbed through the skin and the efficacy of the drug product could vary broadly (21).

Given the problems associated with infusion techniques and transdermal routes of administration, it is crucial to develop a more convenient and practical means of achieving CDS and applying to the most of the PD patient groups.

Rotigotine is a non-ergoline agonist of dopamine D3/D2/D1 receptors for the therapy of PD which has similar structure to dopamine (22). It is a lipid-soluble agonist with a molecular weight of 315. Due to an extensive first-pass effect and relatively short plasma half-life (5–7 h) (23), rotigotine shows a very low bioavailability through oral administration.

The present study was designed to prepare a novel long-acting injection of rotigotine loaded microspheres (RoMS) to avoid first-pass metabolism and to provide an alternative of CDS drug delivery system, e.g., once every 2 weeks. Although RoMS could be effective in the therapy of early PD, L-DOPA was still an inevitable supplementation for most patients with PD (20,24). An important issue was raised whether the CDS therapy in combination with L-DOPA could delay or reduce the dyskinesia development following the addition of L-DOPA. For this reason, a further experiment was conducted to compare the effects of RoMS in combination with L-DOPA on dyskinesia development and therapeutic efficacy to L-DOPA monotherapy. Additionally, the effect of the administration mode of rotigotine in the combination treatment on dyskinesia was evaluated.

MATERIALS AND METHODS

Materials

PLGA 7525 2A (lactide/glycolide ratio, 75/25; Mw, 14,000), 7525 4A(lactide/glycolide ratio, 75/25; Mw, 52,000), 5050 2A (lactide/glycolide ratio, 50/50; Mw, 16,000) and 5050 5A (lactide/glycolide ratio, 50/50; Mw, 75,000) were purchased from Lakeshore Biomaterials, USA. Rotigotine was obtained from Shandong Luye Pharmaceuticals (Yantai, China). Polyvinyl alcohol (PVA; Mw 13,000–23,000; 87–89 % hydrolyzed), L-DOPA, 6-OHDA, benserazide, apomorphine, desipramine hydrochloride and ascorbic acid were all purchased from Sigma Aldrich Chemicals, USA. HPLC grade acetonitrile were supplied by Merck Specialities Private Ltd (USA). All other reagents used were analytical grade.

Animals

Male Sprague–Dawley rats (220–260 g) obtained from the Experimental Animal Center of Shandong Luye

Pharmaceutical (Yantai, China) were employed in the *in vivo* evaluation. All animals had free access to water and food was limited to 15 g per day. All the investigations were carried out in accordance with 'Principles of Laboratory Animal Care' (NIH publication no. 85–23, revised 1985) and Experimental Animal Research Committee at Yantai University.

Preparation of RoMS

RoMS were prepared by an oil-in-water emulsion/solvent evaporation technique for that rotigotine and PLGA can be easily dissolved in methylene chloride. 0.30 g (or 0.35 g) of rotigotine and 0.70 g (or 0.65 g) of PLGA polymer or polymer blends were dissolved in 5 mL methylene chloride. The solution was added slowly to 500 mL 0.5 % PVA solution and homogenized (by YIKA homogenizer, China) at various rates (1500–2000 rpm) for 2 min to obtain an emulsion with similar particle size. The resulting emulsion was stirred at 400 rpm for evaporation of methylene chloride. The solidified microspheres were collected with a 10 μm sieve and washed by 500 mL water for three times. The wetted microspheres were then freezing dried. To obtain desired formulation, PLGA 7525 4A was combined in various proportions with 5050 2A as detailed in Table I.

Characterization of the Microspheres

A laser particle size analyzer (Mastersizer 2000, UK) was used to determine the particle diameter of the microspheres. Approximately 50 mg of the microspheres were dispersed in 50 mL of distilled water and sized after mixing for 10 s.

Morphology of the microspheres was studied by scanning electron microscopy (SEM) (JSM-840, JEOL, Japan). The microspheres were fixed on aluminum studs and coated with gold using a sputter coater. And then the morphology was scanned.

The amount of rotigotine encapsulated in the microspheres was determined by HPLC method. Approximately 20 mg of RoMS were dissolved in 1 mL acetone and 0.01 M HCl was added up to 25 mL. The suspension was filtered (0.45 μm) and analyzed by HPLC using UV detection at 223 nm with a mobile phase of acetonitrile and 0.3 % H_3PO_4 (34:66, pH 2.5). The drug loading was determined and the encapsulation efficiency (EE) were calculated as: EE (%)=(drug loading/theoretical drug loading)×100.

The practical yield (%) of RoMS was calculated as the following:

$$\text{Practical yield (\%)} = \frac{\text{Weight of dried RoMS}}{\text{Weight of (rotigotine + PLGA)}} \times 100\%$$

In Vitro Release

In vitro release studies were conducted with a modified USP apparatus 4 (CE7 smart, Sotax, Switzerland). Flow-through cells (12 mm diameter) packed with glass beads were used in a closed system of 37 °C. Approximately 30 mg of RoMS were scattered in the cells and the dissolution medium was 250 mL of 0.1 M phosphate buffered saline (PBS, pH 7.4) with 0.1 % (*w*/*v*) SDS with a flow rate of 8 mL/min. Samples of 10 mL were withdrawn at the following intervals (3 h and 1, 2, 4, 6, 8, 10, 12, 14 days) and replaced with fresh media. The samples were analyzed by HPLC method as described above. All measurements were conducted in triplicate.

Pharmacokinetics Study

RoMS was suspended in saline containing 1.0 % carboxymethyl cellulose sodium (10 mg rotigotine/mL) prior to injection. Rats (n=6 per formulation) were injected intramuscularly (im) at a dose of 10 mg/kg/14d. Blood samples were collected at the following intervals: 1, 3, 6 h and 1–14 days. The samples obtained were immediately centrifuged at 3000 rpm and the plasma was stored at −20 °C before analysis. Rotigotine was analyzed by LC-MS/MS as described previously (25): The HPLC system (Agilent 1100 series) consisted of a binary pump, an autosampler and a

Table I Characteristics of the Prepared Rotigotine Loaded Microspheres Formulations

Formulation	PLGA	Homogenization rate (rpm)	Drug loading (%)	EE (%)	Practical yield (%)	Particle size (μm)
A	5050 2A	1500	26.7	88.9	70.1	70.4
B	5050 5A	2000	26.8	89.2	75.4	80.3
C	7525 2A	1500	26.4	87.9	72.0	69.6
D	7525 4A	2000	27.1	90.3	73.3	78.4
E	7525 4A	2000	30.5	87.1	73.7	70.1
F	7525 4A:5050 2A (9:1)	2000	26.8	89.1	69.9	74.5
G	7525 4A:5050 2A (8:2)	1800	26.4	87.8	68.6	72.9
H	7525 4A:5050 2A (7:3)	1600	27.0	90.2	71.7	71.5

Zorbax C18 column. The mobile phase was acetonitrile-1 mM ammonium acetate (75:25, *v/v*) at a flow-rate of 0.30 mL/min. The detection was performed on the TSQ Quantum Access mass spectrometer (Thermo, USA) operated in the positive ion mode. Multiple reaction monitoring at unit resolution involved transitions of the protonated forms of rotigotine at m/z 316.2→147.1 and diazepam at m/z 285.4→193.1. Optimized MS conditions were as follows: curtain gas, gas 1 and gas 2 (all nitrogen) 15, 50 and 50 psi respectively; ion spray voltage 5500 V; source temperature 500 °C; declustering potentials 50 V for rotigotine and 160 V for diazepam; collision gas was set at 3 psi.

The brain microdialysis experiments were conducted according to previous described methods (26). Briefly, under anesthesia, the CMA/12 guide cannula (CMA Microdialysis, Sweden) was implanted stereotaxically into the left striatum of the rat (A=0.2 mm, L=3.0 mm, and V=−4.2 mm from bregma and the dural surface) and secured with dental acrylic. After a recovery of 4–6 days, the CMA12 microdialysis probe with an active membrane surface of 4 mm (cut-off 20 kDa) was inserted into the striatum via guide cannula and perfused with Ringer's solution (145 mM NaCl, 1.2 mM $CaCl_2$, 2.7 mM KCl, 1.0 mM $MgCl_2$, pH=6.5) at a flow rate of 1 μL/min. Samples were collected every 30 min. After a stabilization period of 1.5 h, RoMS suspension was injected (10 mg/kg/14d, im) and sampling continued for 4 h on each test day. Rotigotine was analyzed using LCEC (26). The HPLC system (Agilent 1100 series) consisted of a binary pump, an autosampler and a Luna C18 silica microbore column (150×2 mm, 3 μm, Phenomenex, USA). An electrochemical detector was equipped with 5040 electrode (ESA, USA). The potential of the glassy carbon electrode was set to +850 mV *versus* the Ag/AgCl/3 M NaCl reference electrode. The mobile phase consisted of 50 mM $Na_2HPO_4{\cdot}2H_2O$, 1.7 mM sodium octylsulfonate (pH 4.5) and 35 % acetonitrile (*v/v*) with a flow-rate of 0.1 mL/min. The *in vitro* and *in vivo* calibration of the microdialysis probe experiments were carried out by retrodialysis method as described by Jan Kehr (26). Briefly, the microdialysis probe was implanted into the brain as described above and the perfusate was Ringer's solution containing rotigotine at 10 ng/mL. Samples were collected every 30 min after a stabilization period of 1.5 h. The *in vivo* recovery of rotigotine was calculated as the following: $R_{in\ vivo}=1-C_{dialysis}/C_{perfusate}$. The *in vivo* recovery was calculated by a different set animals because that rotigotine residue in the brain by the retrodialysis method would probably affect the quantitative determination of the drug and it could avoid the damage from probe implantation on rats tested. *In vitro* experiments were conducted analogously to the *in vivo* recovery except that the microdialysis probe was immersed into Ringer's solution. The *in vitro* and *in vivo* recovery of rotigotine was 71.6±4.3 % (n=3) and 33.2±5.9 % (n=6) respectively. The *in vitro* recovery was conducted before and after daily sampling and it was constant during the whole test period by careful processing, indicating the consistency of the *in vivo* recovery.

Severe Unilateral 6-OHDA Lesion

The animals were lesioned by the method described by Przedborski (27) for unilateral nigrostriatal degeneration. The animals were first anesthetized by pentobarbital-sodium (45 mg/kg, ip). To prevent noradrenergic neurons against damage from 6-OHDA, animals were pretreated with desipramine hydrochloride (20 mg/kg, ip) 30 min before injection of 6-OHDA. 6-OHDA (4 μg/μL in 0.9 % saline, containing 0.04 % ascorbic acid) 4 μL was then injected into the left medial forebrain bundle via a stereotaxic frame (stoelting, USA) according to the following coordinates: x=−4.0 mm; y=+1.65 mm and z=−8.0 mm (from the Bregma) (27,28). Sham-operated rats received the same volume of saline containing 0.04 % ascorbic acid to rule out the factors of mechanical injury and no rotation was seen in sham-operated rats. After 3 weeks of 6-OHDA injections, animals were evaluated for contralateral rotation induced by apomorphine (Apo, 2 mg/kg, ip). Rats that performed more than 5 rotations/min were included in experimental groups. Rats suffering such lesions will exhibit rotation to the contralateral upon dopaminergic treatment due to hypersensitization of the dopamine receptors of the lesioned site (3).

Pharmacodynamics Study of Treatment Groups

The experiment was carried out in two sets of investigations (Fig. 1). In the first experiment (Fig. 1a), 6-OHDA-lesioned rats were divided into four treatment groups randomly. Each group of animals was treated for 14 days. Group 1 (n=6) received vehicle (1.0 % carboxymethyl cellulose sodium, 0.2 mL/rat/14 d, im). Group 2 (n=8) were treated with RoMS (10 mg/kg/14 d, im), Group 3 (n=8) pulsatile L-DOPA (corresponding to 200/50 levodopa/benserazide, 20 mg/kg/d, po), and Group 4 (n=9) RoMS (10 mg/kg/14 d, im) and pulsatile L-DOPA (20 mg/kg/d, po) combination. The second experiment (Fig. 1b) was designed to determine whether RoMS in combination with pulsatile L-DOPA could modify already established dyskinesia. In the first phase, the PD rats received a dose of L-DOPA (20 mg/kg/d, ip) for 7 consecutive days for LID. Then the animals were divided randomly into five treatment groups. Each group received one of the following treatments for 2 weeks. Group 1 (n=6) received vehicle (1.0 % carboxymethyl cellulose sodium, 0.2 mL/rat/14 d, im). Group 2 (n=9) received L-DOPA (20 mg/kg/d, po), Group 3 (n=9) RoMS (10 mg/kg/14d, im) and L-DOPA (20 mg/kg/d, po)

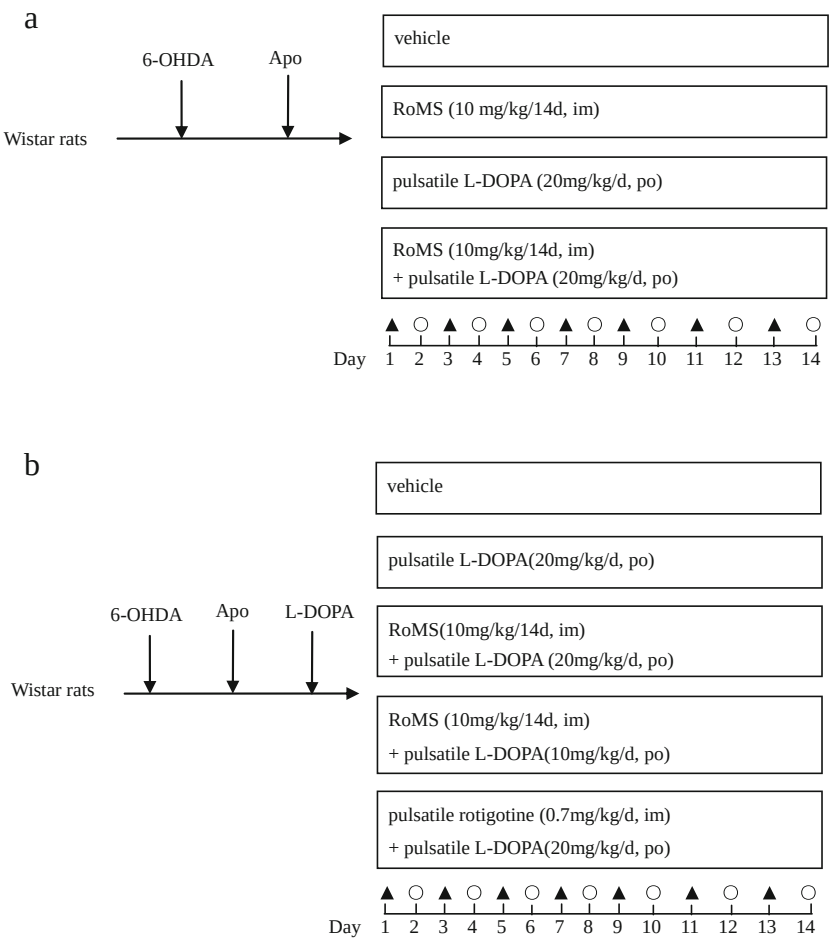

Fig. 1 Schematic of the experimental performance of (**a**) first set and (**b**) second set of investigations. Rotation activity tests were carried out on days 1, 3, 5, 7, 9, 11 and 13 (*black triangles*) and dyskinesia on days 2, 4, 6, 8, 10, 12 and 14 (*open circles*).

combination, Group 4 (n=9) RoMS (10 mg/kg/14d, im) and L-DOPA (10 mg/kg/d, po) combination, and Group 5 (n=8) received the combination treatment of rotigotine injection, a solution of hydrochloride salt of rotigotine (corresponding to free base of rotigotine 0.7 mg/kg/d, im), and L-DOPA (20 mg/kg/d, po).

The rotation activity was assessed on drug treatment days 1, 3, 5, 7, 9, 11, 13 for 2 h and AIMs on drug treatment days 2, 4, 6, 8, 10, 12, 14 for 2 h.

Assessment of Rotation Activity

The rats were placed separately into a circular cardboard chamber. The contralateral rotation numbers were counted manually every 5 min and recorded for 2 h after a 5-min period adaptation. Each time a rat turned around for 360° in one direction was counted as one rotation.

Assessment of Dyskinesia

Dyskinesia was assessed using a glass cylinder (with the diameter of 17 cm) and manually scored by an observer blinded to the protocol. Three types of AIMs were rated separately including masticatory, forelimb and axial dyskinesia using scales as followings: 0 = absent, 1 = mild, 2 = moderate, 3 = marked, 4 = severe. The AIMs was evaluated every 30 min for 2 h and the score recorded per rat challenge ranged from 0 to 48.

Statistical Analysis

All data were reported as means ± standard deviation (SD). Statistical analysis between groups was determined by one-way analysis of variance (ANOVA) followed by Dunnett's test. A value of $P<0.05$ was considered as statistically significant and $P<0.01$ was considered as highly significant.

RESULTS

In Vitro Characterization of RoMS

The physicochemical characteristics of RoMS are summarized in Table I. The drug loadings and particle size for all formulations were similar and a high EE of about 90 % was obtained. The practical yield of RoMS is about 70 %. SEM analysis showed that the surface of the microspheres was spherical, smooth and nonporous. The *in vitro* cumulative

release profiles of rotigotine from all formulations are shown in Fig. 2. As expected, the microspheres prepared from PLGA 5050 2A, 7525 2A and 5050 5A showed rapid drug release, while a slow release which was sustained for 14 days was found in the case of 7525 4A microspheres. A modification in the rotigotine release profiles was obtained with the polymer blends formulation.

Pharmacokinetics Study of Rotigotine Levels in Plasma

The plasma concentration-time profiles of rotigotine after im injections of RoMS in rats are shown in Fig. 3. The drug release of Formulation A (5050 2A) showed a high initial burst release of approximately 27 % within 24 h. A sustained drug release with a lag phase from 2nd to 6th day was detected in the case of Formulation D (7525 4A). Although PLGA 5050 5A has a high molecular weight, Formulation B only exerted a sustained-release of rotigotine for 10 days due to a low *Tg* of the polymer. A smooth release within 14 days was achieved by addition of 30 % of 5050 2A into 7525 4A (Formulation H) in which the lag period was significantly modified: a stable mean plasma level of 4.11±1.59 ng/mL for near two weeks with a C_{max} of 6.27±2.35 ng/mL on 6th day and a C_{min} of 1.39±0.13 ng/mL at 14th day after the im administration.

Pharmacokinetics Study of Rotigotine Levels in Brain Microdialysates

Rotigotine concentration in brain microdialysates (corrected by *in vivo* recovery of 33.2±5.9 %) after the administration of RoMS (Formulation H) is shown in Fig. 4. Rotigotine can be detected in the dialysate in the first 1 h after the RoMS administration. A peak concentration of 8.16±0.95 ng/mL

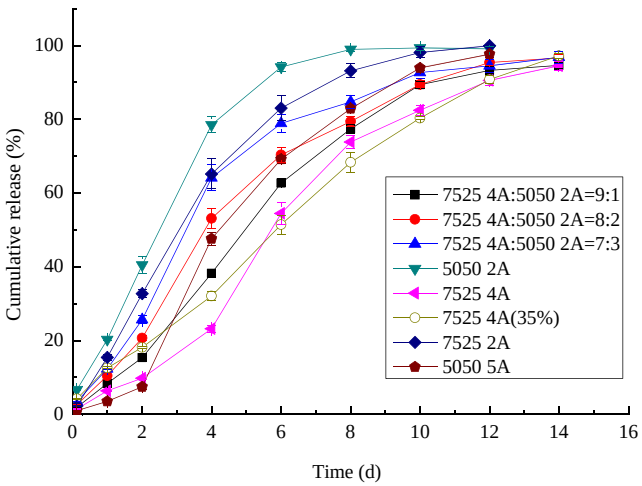

Fig. 2 *In vitro* release of rotigotine loaded microspheres. Each point represents mean±SD (n=3).

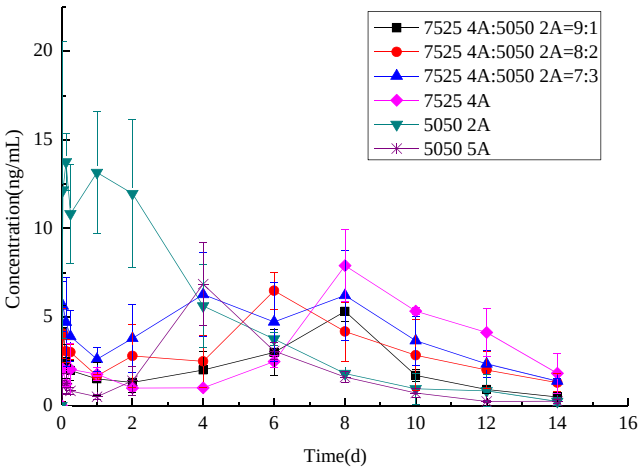

Fig. 3 Plasma concentration *vs.* time curves for rotigotine after administrations of RoMS. Each point represents mean±SD (n=6).

(corrected by *in vivo* recovery) was obtained on day 8 and a constant and stable level of extracellular rotigotine was maintained at 3.94±2.06 ng/mL (corrected by *in vivo* recovery).

Pharmacodynamic Effects of Experiment I

Contralateral Rotations (Fig. 5a)

RoMS (Group 2) improved constant contralateral rotations and reached a maximum of 45±15 circles/5 min on the 3rd test day and maintained constant until 11th test day and declined to 13±4 circles/5 min on 13th day. Pulsatile L-DOPA induced an significant increase of contralateral rotation number on each test day in Group 3, it might be due to a high activity of L-DOPA and a high plasma concentration of the drug with 2 h after oral administration (29). RoMS and pulsatile L-DOPA combination group (Group 4) resulted in also an increase of rotational activity compared to that of Group 2, but not statistically different

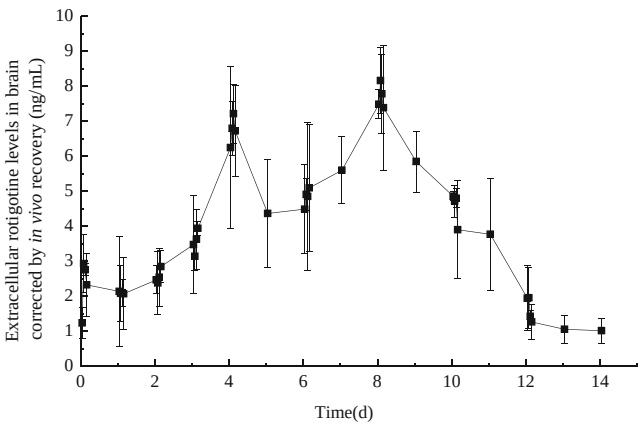

Fig. 4 Rotigotine levels in brain microdialysates following administration of RoMS (corrected for recovery). Each point represents mean±SD (n=4).

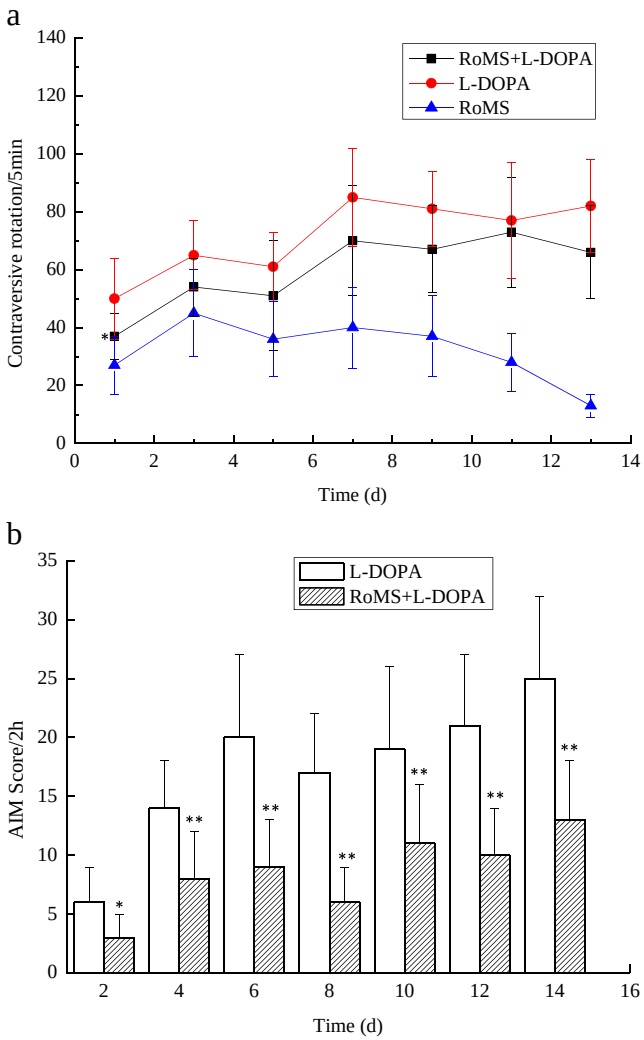

Fig. 5 Behavior effects of 6-OHDA-lesioned rats treated with RoMS, pulsatile L-DOPA, or concomitant of RoMS and pulsatile L-DOPA during the first set of investigations: (**a**) contraversive rotation. *$P<0.05$ compared to the pulsatile L-DOPA group; (**b**) AIMs. *$P<0.05$; **$P<0.01$.

compared to Group 3 [$P>0.05$, except on the first test day ($P=0.023$)]. The vehicle group showed no contralateral rotation activity.

AIMs (Fig. 5b)

As was expected, no incidence of AIMs was observed in Group 2 and vehicle group at any time tested. Pulsatile L-DOPA induced mild to moderate AIMs in Group 3. In contrast, RoMS and pulsatile L-DOPA combination (Group 4) showed a significantly lower score of AIMs during the test days than that of Group 3 [$P<0.01$, except on the 2nd test day ($P<0.05$)].

Pharmacodynamic Effects of Experiment 2

First L-DOPA Administration for the LID Rat Model

The rats were evaluated for the effect of daily L-DOPA on dyskinesia in the cylinder test. L-DOPA, administered for 7 continuous days, induced marked to severe AIMs in all rats at 7th day of treatment (data not shown) which indicated that the LID rat model was successively obtained. Then the rats were divided into five treatment groups randomly.

Second Treatment with Therapeutic Effects

Contralateral Rotations (Fig. 6a). Following the administration of pulsatile L-DOPA, contralateral rotations remained high during whole test period in Group 2 animals. The animals in Group 3 of RoMS and pulsatile L-DOPA combination showed a similar rotation activity to that of Group 2 ($P>0.05$ on each test day). When the dose of L-DOPA in the combination treatment was reduced by 50 % (to 10 mg/kg/d) in Group 4, the rotational activity was followed by a moderate decrease and no significant difference was observed between Group 3 and 4 except on 14th test day ($P>0.05$, $P=0.021$ on 14th test day). Pulsatile rotigotine and pulsatile L-DOPA combination (Group 5) caused also similar contralateral rotations to that of Group 2. No contralateral rotation was observed in the vehicle group (Group 1).

AIMs (Fig. 6b). Pulsatile L-DOPA (Group 2) induced marked to severe AIMs over the whole treatment period, while RoMS and pulsatile L-DOPA combination (Group 3) led to a significant decrease of AIMs intensity comparing to Group 2 [$P<0.01$, except on 2nd test day ($P<0.05$)]. An unexpected finding was, in the case of a reduced dosage of L-DOPA in the combination treatment (Group 4), the AIMs tended to decline significantly over the test period [$P<0.01$, except on 2nd test day ($P<0.05$), *versus* Group 3], though the contralateral rotations were not significant different between the two groups. Marked to severe dyskinesia was observed in Group 5 animals which was similar to Group 2. There is no incidence of AIMs in the vehicle group.

Overall, contralateral rotations produced by each treatment (Group 2, 3 and 5) was comparable while treatment of RoMS and pulsatile L-DOPA combination induced significant less intense dyskinesia compared to the other two groups on the LID rats. When the dosage of L-DOPA of the combination treatment was reduced by 50 %, the

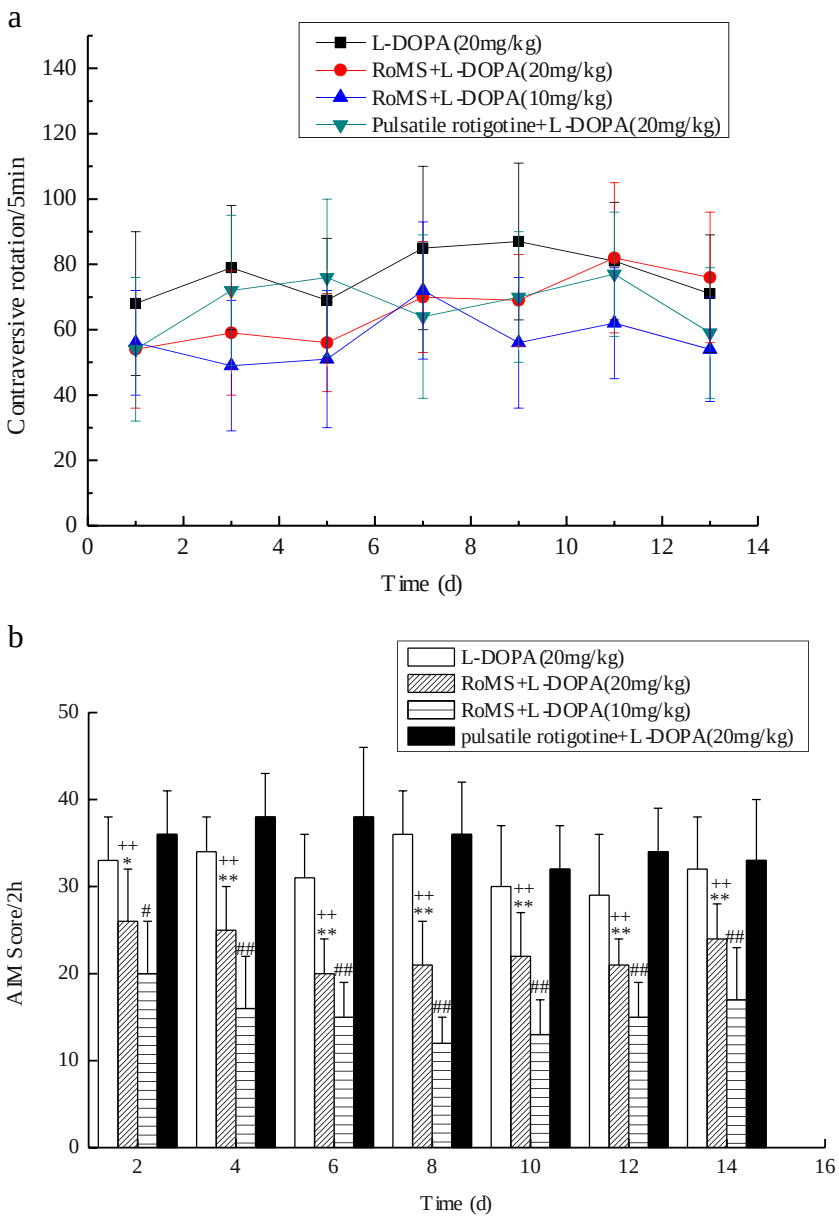

Fig. 6 Behavior effects of 6-OHDA-lesioned rats treated with pulsatile L-DOPA (20 mg/kg/d), RoMS and pulsatile L-DOPA (20 mg/kg/d), RoMS and pulsatile L-DOPA (10 mg/kg/d) or pulsatile rotigotine and pulsatile L-DOPA (20 mg/kg/d) during the second set of investigations: (**a**) contraversive rotation; (**b**) AIMs. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the pulsatile L-DOPA group; $++P < 0.01$ compared to the pulsatile rotigotine and pulsatile L-DOPA group; $\#P < 0.05$; $\#\#P < 0.01$ compared to the RoMS and pulsatile L-DOPA (20 mg/kg/d) group.

rotation activity decreased moderately while the AIMs decreased significantly.

DISCUSSION

Dyskinesia is always a focus of concern in the long-term treatment of PD. CDS strategy has been employed to prevent the dyskinesia induction in early PD, however, ameliorating established dyskinesia as well as maintaining therapeutic efficacy in mild and advanced PD still remain to be a challenge. The purpose of the present investigation was to examine the effects of RoMS and pulsatile L-DOPA combination treatment on the therapeutic effects and especially on the potentiality to delay or reduce dyskinesia development. The mono-treatment with pulsatile L-DOPA was used as reference because of its effects of pro-dyskinesogenics which were well described previously (30).

Considering the extensive first-pass effect of rotigotine and the requirement of long-term medication for PD patients and CDS therapy on PD, rotigotine is formulated as extended-release microspheres for injection. The physicochemical properties of rotigotine, such as lipid-soluble and low molecular weight, enable it to be entrapped into PLGA microspheres with a high efficiency by an emulsion/solvent evaporation technique. It should be noted that the drug release profiles of microspheres can be modified by the blends of PLGA polymers with varied LA/GA ratio and molecular weights which led to different *Tg* and biodegradation. The drug release profiles of the microspheres from

blended polymers should be presumed that the initial release could be mainly attributed to the more hydrated polymer of 5050 2A, and the subsequent release was due to the higher composition of 7525 4A in the matrix.

The idea of CDS has emphasized on not only the importance of continuous drug delivery but also the continuous and steady drug levels in both plasma and brain striatum (31). The pharmacokinetics study of RoMS showed that both the plasma and striatal levels of rotigotine were constant and stable up to 14d. The steady-state brain extracellular rotigotine levels monitored by microdialysis indicated that RoMS could result in continuous striatal dopamine receptors stimulation (32). The rotigotine concentration in brain is comparable to that observed in plasma [protein binding: 92 % (23)] suggesting that a high level of rotigotine passed through the blood–brain barrier due to the lipophilicity of the drug (33). Further, administration of RoMS increased constant and steady contralateral rotations while no incidence of dyskinesia. These results suggest that RoMS will possess the benefits of CDS, reduce risk of dyskinesia and delay introduction of L-DOPA in the treatment of early PD.

The results of contralateral rotations in this study indicated that the mono-treatment of pulsatile L-DOPA and the combination treatment of rotigotine (pulsatile or continuous) and pulsatile L-DOPA showed almost equivalent therapeutic benefits in the rat studies. However, RoMS and pulsatile L-DOPA combination showed an advantage of reducing dyskinesia whether established or not, compared to pulsatile L-DOPA monotherapy, which was a significant concern of this investigation. In the first experiment set, the combination of RoMS and L-DOPA led to less intensity of dyskinesia than mono L-DOPA, suggesting that RoMS could prevent the dyskinesia development following the introduction of L-DOPA. It appears that the initiating treatment manner before the addition of L-DOPA can affect the development of dyskinesia. In addition, a similar trend was observed in the dyskinesia change on LID rats. One possible explanation was that in the combination treatment, RoMS could provide a CDS background which would act as a buffer against the fluctuations occurring in pulsatile L-DOPA stimulation to some extent (34). In early PD, sufficient dopaminergic neurons hold the ability to store dopamine supplied by exogenous L-DOPA to maintain dopamine levels relatively normal. With the progression of PD, the dopamine terminal degeneration was severe and the buffering capacity for fluctuations L-DOPA was lost gradually (35). This may be the reason why there is less dyskinesia observed in the first experiment set than that in the second set.

There is no additional anti-parkinsonian effect found with the addition of RoMS in the combination treatment with L-DOPA in the animal studies. This might owe to that the baseline of rotational activity by L-DOPA has already reached to a maximum level.

It is necessary to point out that the decrease of L-DOPA dose in combination treatment resulted in significant decrease in dyskinesia. Although it is generally considered that high L-DOPA levels induce increased dyskinesia, a significant finding is that only mild decrease in rotation activity has been observed. The finding suggests that it is feasible to reduce the severity of dyskinesia and maintain therapeutic effects by decreasing the dose of L-DOPA in the combination treatment. It also seems likely that L-DOPA can be added with dose-escalation with the progression of PD in the presence of CDS to delay the dyskinesia development which needs further investigation.

Previous study (36) showed that little or no AIMs was induced by pulsatile or continuous rotigotine on the 6-OHDA-lesioned rats. However, in the present study, RoMS and L-DOPA combination induced significantly less severity of dyskinesia than pulsatile rotigotine and L-DOPA combination. This finding suggested that when L-DOPA was administrated in combination with a dopamine agonist, the administration mode of the dopamine agonist influenced the expression of dyskinesia. The CDS background produced by RoMS may play an important role in reducing the dyskinesia intensity in the combination treatment.

CONCLUSION

RoMS can provide a continuous delivery of rotigotine with small fluctuations of drug levels in plasma and brain striatum during dosing intervals in rats and can also produce stable and steady efficacy in the PD rats which lasts for about 14 days, those results enabled RoMS to be a potent alternative of CDS in the treatment of early PD. RoMS and pulsatile L-DOPA combination treatment could reduce the dyskinesia development or the intensity of the established dyskinesia and maintain therapeutic efficacy compared to pulsatile L-DOPA monotherapy. The results indicated the potential advantage of the combination treatment on the mild and advanced PD. The CDS therapy produced by RoMS combined with L-DOPA would probably be a more convenient and practical means for the treatment of mild and advanced PD. Our results are obtained on the basis of studies in 6-OHDA-lesioned rats, which is required to be verified by clinical trials.

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